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Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru

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During an outbreak of tegumentary leishmaniasis that developed in the 1990s in the Eastern Andean valley of Huanuco, Peru, the coexistence of Andean (uta) and sylvatic leishmaniases was suspected for ecological and geographical reasons, and sympatric sampling was carried out. Seven human isolates of *Leishmania* were characterized by multilocus enzyme electrophoresis, random amplification of polymorphic DNA and molecular karyotyping. The three methods identified 3 isolates as *L. braziliensis*, and 4 isolates as putative hybrids with characters of *L. braziliensis* and *L. peruviana*. Data from Huanuco are compared to previous results from other areas endemic for uta. Biological and epidemiological implications are discussed.

Key words: *Leishmania braziliensis*; *L. peruviana*; Epidemic; Recombination; Enzyme Electrophoresis; RAPD; Molecular karyotype

Introduction

Two forms of tegumentary leishmaniasis are endemic in Peru. Sylvatic leishmaniasis is found in the Amazonian forest, and is characterized by florid lesions leading to metastatic mucosal involvement (espundia) in < 10% of the cases (Llanos-Cuentas, 1991). Andean leishmaniasis (uta) is prevalent in Western Andean and inter-Andean valleys, and is generally benign (Herrer, 1962; Lumbreras and Guerra, 1985; Guerra, 1988), but various degrees of severity however have been documented recently (Llanos-Cuentas, unpublished data), including mucosal involvement by contiguity as distinct from metastatic espundia (Llanos-Cuentas, 1991 and unpublished data). As a consequence of such differences in clinical risks, recommended therapies might differ for Andean (local treatment) and sylvatic (systemic chemotherapy) leishmaniases.

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Abbreviations: Deoxyribonucleic acid (DNA), Multilocus Enzyme Electrophoresis (MLEE), Random Amplification of Polymorphic DNA (RAPD), Orthogonal Field Alternated Gel Electrophoresis (OFAGE), Kilobases (kb), Unweighted Pair-Group Method with arithmetic Averages (UPGMA).

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It has been suspected for years that Andean and sylvatic leishmaniases might be encountered sympatrically due to human migration and, in some areas, to geographical continuity between the Andes and the forest (Guerra, 1988). These areas need to be identified for specific local health strategies. Clinical polymorphism requires intrinsic identification of the etiological parasites, namely *L. braziliensis* and *L. peruviana*.

Both parasites are genetically very similar (Romero et al., 1987; Lopez et al., 1988; Reiner et al., 1989) but they are discriminated by isoenzyme analysis (one enzyme, MPI: Arana et al., 1990; Guerrini, 1993) and by molecular karyotyping (size difference for one specific chromosome: Dujardin et al., 1993 a,b). Moreover, distinct geographical populations were found recently within *L. peruviana* in the Western Andean valleys (Dujardin et al., 1993 b; Guerrini, 1993). Such characteristics might allow epidemiological tracking.

For ecological reasons, sympatric co-existence of both species was questioned during an outbreak of cutaneous leishmaniasis in the Eastern Andean valley of Huanuco in the 1990s (Llanos-Cuentas, unpublished data). The Huanuco Valley is situated at the same altitude ($\sim$2000 m) as the western Andean valleys where uta is endemic, is xerophytic and is in direct contact with the Amazonian forest.

Seven human isolates were collected over a short period of time from the same area in the Huanuco Valley. Characterization of these isolates was performed by multilocus enzyme electrophoresis, random amplification of polymorphic DNA and molecular karyotyping combined with hybridization with three DNA probes. The results indicate the sympatric presence of *L. braziliensis* and of putative hybrids with genetic characters from *L. braziliensis* and *L. peruviana* in the Huanuco Valley.

Materials and Methods

Parasites.

Seven isolates (Table 1) were obtained from patients with cutaneous lesions. One patient, from which stock LC1408 was isolated, presented an additional mucosal lesion. According to the information available, these patients were born, lived and were infected in one and the same district of Huancapallac in the Eastern Andean valley of Huanuco (Fig. 1), at the border between the biogeographical units (according to the classification of Lamas, 1982) of Huanuco (open lower montane, 500–2000 meters above sea level) and Huallaga (lower montane forest, 500–1500 meters). Promastigotes for each isolate were subcultivated fewer than 20 times (following isolation) in blood agar medium (Tobie et al., 1950), and were harvested as one and the same pool during late log phase to prepare samples for the three methods of characterization. Seven reference stocks of *L. braziliensis* and *L. peruviana* (see Table 1) were processed similarly.

Multilocus Enzyme Electrophoresis (MLEE).

Fifteen enzyme systems were run in cellulose acetate electrophoresis, namely: aconitase (ACON : EC 4.2.1.3), glucose-6-phosphate dehydrogenase (G6PD : EC 1.1.1.49), glucose phosphate isomerase (GPI : EC 5.3.1.9), glutamate oxaloacetate transaminase (GOT : EC 2.6.1.1), glutamate pyruvate transaminase (ALAT : EC 2.6.1.2), isocitrate dehydrogenase (IDH : EC 1.1.1.42), malate dehydrogenase

TABLE 1

Designation and origin of reference stocks and isolates (C, cutaneous lesions; M, mucosal lesion)

Designation	Origin	Lesion	Code
Reference stocks			
L. braziliensis			
MHOM/BR/75/M2903[a]	Brazil, Para, Carajas	C	1
MHOM/PE/90/FY[b]	Peru, Madre de Dios	–	2
ILLN/BO/84/LPZ595[c]	Bolivia, Alto Beni	–	3
L. peruviana	Province/District		
MHOM/PE/90/HB31	Huancabamba/Huancabamba	C	4
MHOM/PE/84/LH115	Recuay/Pararin	C	5
MHOM/PE/90/LCA01	Lucanas/Sancos	C	6
MHOM/PE/90/LH807	Canta/San Buenaventura	C	7
L. sp from Huanuco	District, Locality		
MHOM/PE/91/LC1407	Huancapallac, Limapampa	C	8
MHOM/PE/91/LC1408	Huancapallac, Limapampa	C/M	9
MHOM/PE/91/LC1409	Huancapallac, Limapampa	C	10
MHOM/PE/91/LC1412	Huancapallac, Limapampa	C	11
MHOM/PE/91/LC1417	Huancapallac, Limapampa	C	12
MHOM/PE/91/LC1418	Huancapallac, Limapampa	C	13
MHOM/PE/91/LC1419	Huancapallac, Puyac	C	14

Sources: [a]Dr D. Evans (LSHTM, London), [b]Dr S.Revollo (IBBA, La Paz), [c]Dr P. Desjeux (WHO, Genève).

NAD+ (MDH : EC 1.1.1.37), malate dehydrogenase NADP+ or Malic Enzyme (ME : EC 1.1.1.40), mannose phosphate isomerase (MPI : EC 5.3.1.8), nucleoside hydrolases 1 and 2 (NH1: EC 2.4.2.1 and NH2 : EC 2.4.2.*), peptidases 1 and 2 (PEP1 and PEP2 : EC 3.4.4.11 or 13), 6 phosphogluconate dehydrogenase (6PGD: EC 1.1.1.44), and phosphoglucomutase (PGM : EC 2.7.5.1). Electrophoresis and staining procedures were carried out according to Ben Abderrazak et al. (1993).

Random Amplification of Polymorphic DNA (RAPD).

This technique, first described by Williams et al. (1990) and Welsh and McClelland (1990), has been applied to population genetics and to strain characterization of parasitic protozoa, including *Leishmania*, by Tibayrenc et al. (1993). According to the results of the latter authors, we have selected eight 10-bp length primers from the Bioprobe A kit (Table 2). DNA was extracted with phenol-chloroform followed by ethanol precipitation (Sambrook et al, 1989). Conditions of amplification were identical to Williams et al. (1990). Fragment polymorphism was analyzed on ethidium bromide-stained 1.6% agarose gels. DNA molecular weight markers VI and VII (Boehringer Mannheim) were used for sizing DNA fragments.

Molecular karyotyping.

Agarose plugs containing leishmanial chromosomes were prepared for OFAGE as described elsewhere (Van der Ploeg et al., 1984). Electrophoresis set-up and conditions for OFAGE were as described by Dujardin et al. (1987). Resolution of chromosomal bands was achieved with pulses of 65 and 115 s. The karyotype of the reference strain *L. braziliensis* M2903 was used to estimate the size of chromosomal bands.

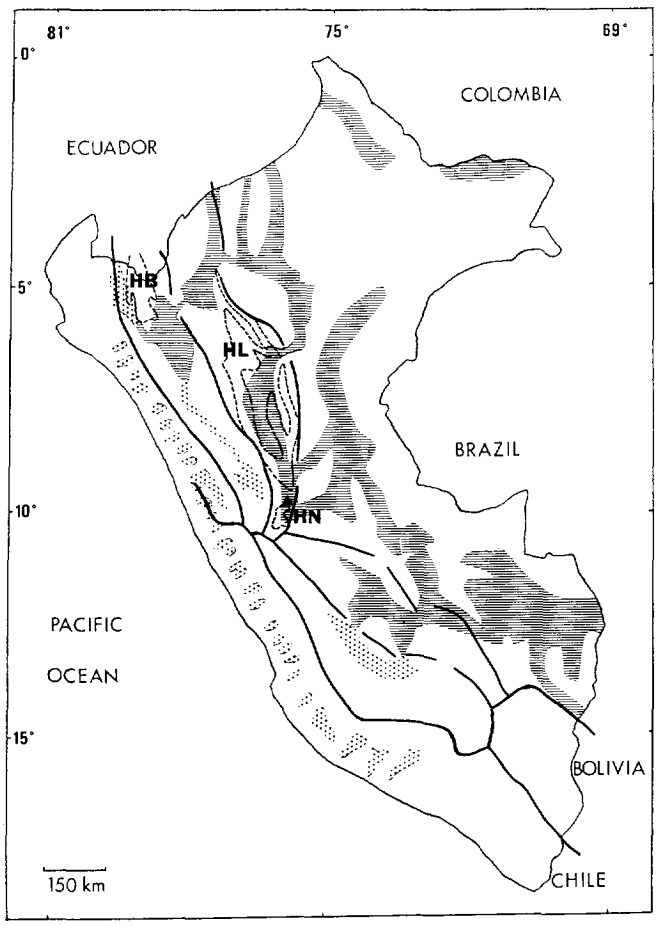

Fig. 1. Map of Peru showing the localization of the valley of Huanuco (HN; triangle = origin of the isolates studied herein) with respect to the areas endemic for uta (dotted) and for sylvatic leishmaniasis (horizontal lines) as described by Guerra (1988). The main mountain ranges are indicated in thick lines. The limits of the biogeographical units are surrounded with dotted lines (HN, Huanuco; HL, Huallaga; HB, Huancabamba).

TABLE 2

Sequence of the eight oligonucleotide primers used for RAPD (Bioprobe, kit A)

A1	CAGGCCCTTC
A2	TGCCGAGCTG
A4	AATCGGGCTG
A5	AGGGGTCTTG
A7	GAAACGGGTG
A10	GTGATCGCAG
A12	TCGGCGATAG
A15	TTCCGAACCC

DNA hybridization.

Chromosomal bands resolved by OFAGE were transferred onto nylon filter (Hybond N, Amersham) according to the manufacturer's instructions. Three random DNA probes derived from a genomic library of *L. braziliensis* M2904 (Dujardin et al., 1993a) were used: pLb-134Sp (subcloned from pLb-134, Dujardin et al., 1994), pLb-168 and pLb-22. They were labelled with ^{32}P dCTP by random prime labelling and hybridized according to the manufacturer's (Amersham) instructions. The final washes after hybridization were performed at high stringency (0.1x SSC, 65°C).

Estimating genetic relationships.

Genetic relationships among the stocks were inferred by computing Jaccard distances (Jaccard 1908) from both MLEE and RAPD data, on all possible stock pairwise comparisons: $D_{ij} = 1 - a/(a + b + c)$ where a = number of bands that are common to the stocks i and j; b = number of bands present in the first genotype and absent in the second; c = number of bands absent in the first genotype and present in the second. From the resulting distance matrix, UPGMA (unweighted pair-group method with arithmetic averages) dendrograms were developed (Sneath et al., 1973), which showed the genetic relationships among the stocks as evidenced either by MLEE or by RAPD.

Results

Multilocus enzyme electrophoresis (MLEE)

The 15 enzyme systems evidenced 16 different putative loci, with two loci for NH1. In the 13 stocks tested (including the reference stocks), 6 loci (IDH, MDH, ME, MPI, NH1-a, NH2) out of 16 were variable (Table 3, polymorphism rate = 0.38), hence overall variability within this sample can be considered as limited, which is confirmed by the maximum and average genetic distances (0.47 and 0.31 +/- 0.13, respectively). In spite of this fact, these 6 variable loci made it possible to individualize 11 different enzyme profiles or zymodemes.

The MPI locus has been shown to be of diagnostic value between *L. peruviana* and *L. braziliensis* (Arana et al., 1990; Guerrini, 1993). According to present results at this locus, 3 isolates (LC1409, LC1412 and LC1417) would be identified as *L. braziliensis*, while the other 4 ones (LC1407, LC1408, LC1418 and LC1419) would be considered as *L. peruviana* (Table 3).

Interestingly, the 4 isolates displaying the *L. peruviana* MPI pattern, when tested for NH2 exhibited the same three-banded pattern that is typical of a heterozygous state for a diploid organism with a dimeric enzyme (Fig. 2). These profiles cannot be explained by a mixture of stocks, for in this last case, the expected profile would present 2 bands only. Moreover, it is worth noting that the two alleles inferred for these putative heterozygous profiles correspond to the alleles considered by Guerrini (1993) as specific respectively for both *L. braziliensis* and *L. peruviana* from Northern Peru on the one hand, and for *L. peruviana* from Southern Peru on the other hand.

With the other enzyme systems considered herein, no diagnostic allele discriminates *L. peruviana* from *L. braziliensis*. Heterozygous patterns are hence not expected in putative hybrids for these enzyme systems, which was indeed the case in the present data.

TABLE 3

Enzyme patterns

Stocks/loci	ACON	ALAT	GOT	G6PD	GPI	IDH	MDH	ME	MPI	NH1–a	NH1–b	NH2	6PGD	PGM	PEP1	PEP2
LH115	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
M2903	5	8	5	4	4	9	17	5	7	2	6	6	6	6	4	5
LC1408	5	8	5	4	4	11	18	7	8	4	6	6–8	6	6	4	5
LC1407	5	8	5	4	4	11	17	6	8	4	6	6–8	6	6	4	5
LC1418	5	8	5	4	4	11	17	5	8	4	6	6–8	6	6	4	5
LC1419	5	8	5	4	4	11	17	7	8	4	6	6–8	6	6	4	5
HB31	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
LPZ595	5	8	5	4	4	11	17	5	7	5	6	6	6	6	4	5
LC1417	5	8	5	4	4	11	17	5	7	4	6	6	6	6	4	5
LC1412	5	8	5	4	4	11	17	5	7	4	6	6	6	6	4	5
LC1409	5	8	5	4	4	11	17	6	7	4	6	6	6	6	4	5
FY	5	8	5	4	4	9	18	5	7	2	6	6	6	6	4	5
LCA01	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5

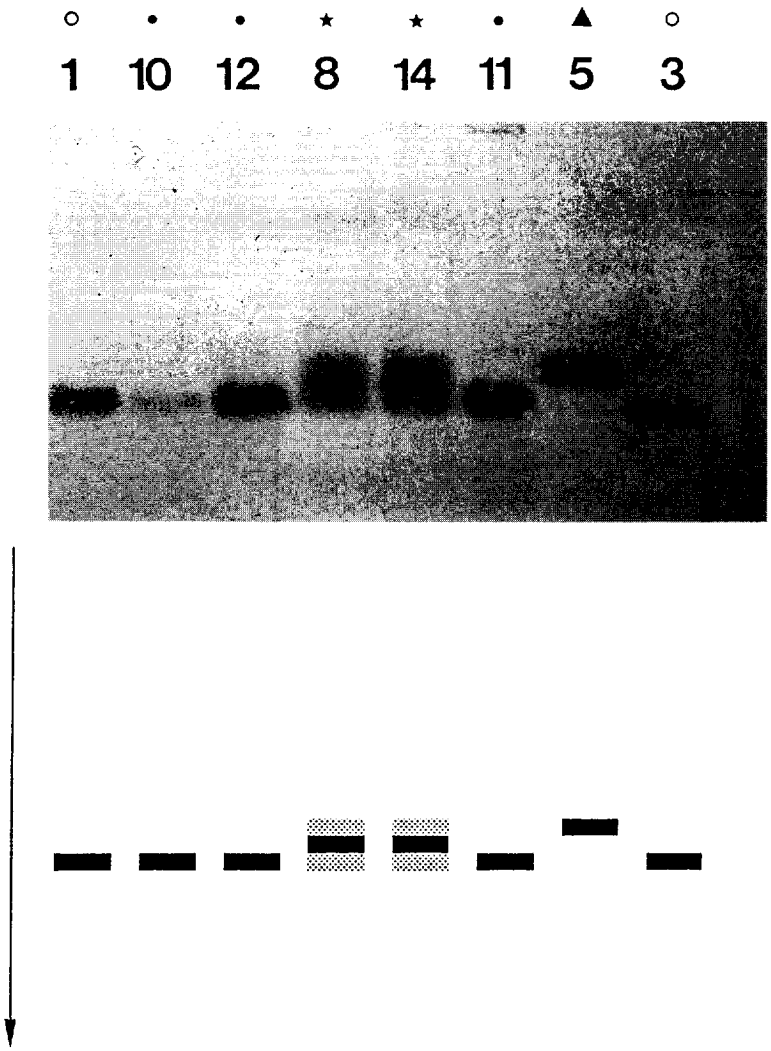

Fig. 2. MLEE gel patterns obtained with NH2: lanes 1,3 (○) and 10, 12, 11 (●) = respectively *L. braziliensis* reference stocks M2903, LPZ595 and *L. braziliensis* isolates from Huanuco LC1409, LC1417, LC1412; lane 5 (▲) = *L. peruviana* reference stock LH115; lanes 8, 14 (★) = heterozygotes from Huanuco LC1407, LC1419.

With all enzyme data processed together, the UPGMA dendrogram (Fig. 3) individualized 4 clusters constituted respectively by (i) the *L. peruviana* reference stocks, (ii) the 4 Huanuco NH2 heterozygotes, (iii) the 3 *L. braziliensis* isolates from Huanuco plus 1 *L. braziliensis* reference stock and (iv) 2 *L. braziliensis* reference stocks. Actually, the four NH2 heterozygotes seem to be equally distant from either *L. peruviana* or *L. braziliensis*: the average genetic distance of heterozygous stocks from either *L. braziliensis* or *L. peruviana* is respectively 0.305 and 0.335 respectively, a statistically non significant difference, as verified by a Student T-test.

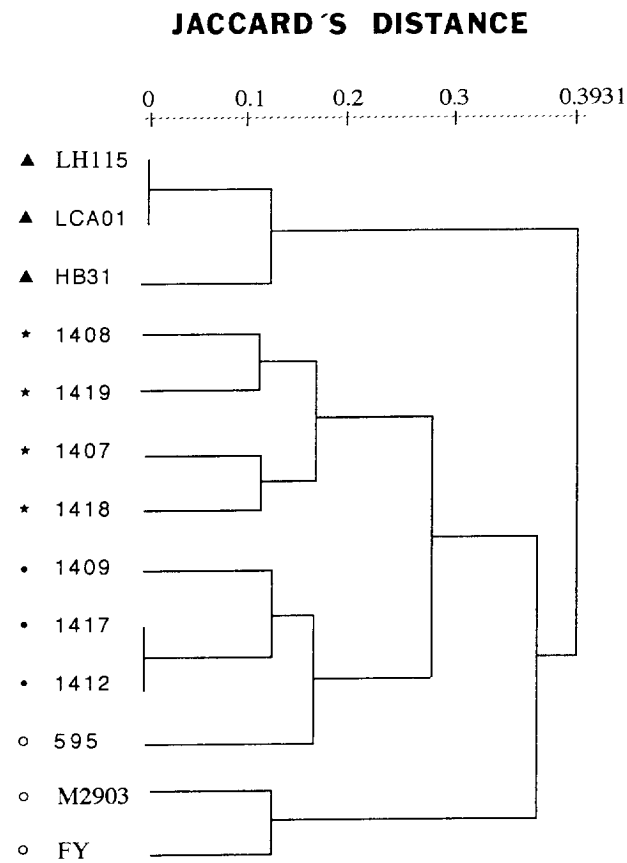

Fig. 3. UPGMA dendrogram built up from the matrix of Jaccard's distances estimated from the isoenzyme data. *L. peruviana* (▲) and *L. braziliensis* (○) are reference stocks; isolates from Huanuco were typed as *L. braziliensis* (●) or NH2-heterozygous (★).

Random Amplification of Polymorphic DNA

Out of 8 primers screened, 4 gave either monomorphic or weakly variable profiles and were not considered any further, nor was a fifth one, for its results were not reproducible. The 3 last primers (namely A2, A4 and A10) provided variable profiles with satisfactory resolution.

Even with these 3 selected primers, overall variability was limited, and few bands diagnostic for either *L. peruviana* or for *L. braziliensis* were recorded.

Here again the 4 NH2-heterozygous stocks exhibited an interesting pattern. The A10 primer amplified a 320-bp fragment shared specifically by the 3 *L. peruviana* reference stocks and by the 4 NH2-heterozygous isolates, while this fragment was observed in none of the 3 *L. braziliensis* reference stocks nor in the 3 *L. braziliensis* isolates from Huanuco (Fig. 4). The A2 primer showed a 1300 bp fragment that was shared by the 3 *L. braziliensis* reference stocks and by the 7 Huanuco isolates (including the 4 heterozygous ones), while this fragment was absent from the 3 *L. peruviana* reference stocks (Fig. 5). Therefore the heterozygous stocks showed RAPD patterns that appeared as a combination of the *L. peruviana* and *L. braziliensis*

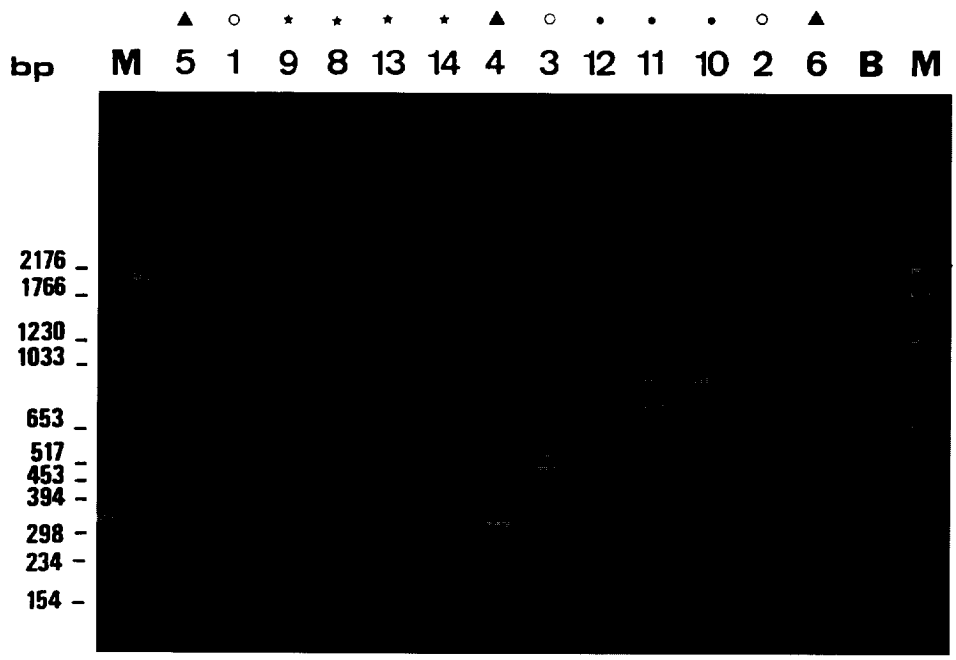

Fig. 4. RAPD gel patterns with primer A10: lane M = size marker, in base pairs; lanes 5,4,6 (▲) = *L. peruviana* reference stocks, LH115, HB31, LCA01; lanes 9,8,13,14 (★) = NH2-heterozygous isolates from Huanuco, LC1408, LC1407, LC1418, LC1419); lanes 1,3,2 (○) and 12,11,10 (●) = respectively *L. braziliensis* reference stocks, M2903, LPZ595, FY and *L. braziliensis* isolates from Huanuco, LC1417, LC1412, LC1409; lane B = blank, negative control without DNA. Arrow: 320-bp fragment specific for *L. peruviana*.

profiles, but not a mere juxtaposition of these profiles, hence they may not be considered as a mix-up of the 2 species.

The UPGMA dendrogram with RAPD data (Fig. 6) was somewhat different from the isozyme dendrogram: the 4 Huanuco NH2-heterozygous isolates (labelled with a star on Fig. 6) clustered together with the *L. peruviana* reference stocks while two of the *L. braziliensis* isolates (LC1412 and LC1417) clustered with the *L. braziliensis* group and the third one (LC1409) fell into a separate group. The average genetic distances between the heterozygous stocks and *L. peruviana* on the one hand, and the heterozygous stocks and *L. braziliensis* on the other hand were 0.279 and 0.415, respectively. This difference is statistically significant at a p level of 10^{-3}, as estimated by Student T-test.

Karyotype analysis

With the same parasites, OFAGE followed by hybridization with three DNA probes discriminated 2 groups, each with a similar karyotype.

Three Huanuco isolates clustered within the first group and they presented (Fig. 7) all the characteristics of *L. braziliensis* (see reference strain M2903, lane 1): (i) a 700 kb pLb-134Sp hybridizing chromosome (Fig. 7a), (ii) a 640 kb pLb-168 hybridizing chromosome (Fig. 7b), and (iii) pLb-22 hybridizing chromosomes $\geqslant$ 1300 kb (Fig. 7c). All the isolates typed isoenzymatically as *L. braziliensis* belonged to this first group.

Fig. 5. RAPD gel patterns with primer A2: lane M = size marker, in base pairs; lanes 5, 6, 4 (▲) = *L. peruviana* reference stocks LH115, LCA01, HB31; lanes 9, 14, 13, 8 (★) = NH2-heterozygous isolates from Huanuco LC1408, LC1419, LC1418, LC1407; lanes 10, 12, 11 (●) = *L. braziliensis* isolates from Huanuco LC1409, LC1417, LC1412; lane 3 (○) = *L. braziliensis* reference stock LPZ595. Arrow: 1300-bp fragment specific for *L. braziliensis*.

The other 4 Huanuco isolates clustered as a second grouping all presenting an heteromorphic (double hybridizing bands) pattern intermediate between *L. braziliensis* and *L. peruviana* (see reference stock in insert, Fig. 7a) with pLb-134Sp *i.e.* one chromosome of 700 kb and another one of 640 kb. Each of the 2 other probes revealed a heteromorphic pattern intermediate between *L. braziliensis* or Northern *L. peruviana* on one hand, and Southern *L. peruviana* on the other hand: 640–700 kb with pLb-168 and, 1300–1150 kb with pLb-22 (Figs 7.a,b). The four NH2-heterozygous isolates belonged to this second group.

Discussion

In Peru, parasites causing the two main tegumentary forms of leishmaniasis, sylvatic leishmaniasis and uta, are encountered in different eco-geographical environments. *L. braziliensis* (sylvatic leishmaniasis) is found in the Amazonian forest, generally at low altitude (Lumbreras and Guerra, 1985). *L. peruviana* (uta) is found in higher altitudes (1300–2800 m above see level) on the highlands of the Western Andean and inter-Andean valleys, usually in xerophytic areas (Herrer, 1962; Lumbreras and Guerra, 1985; Guerra, 1988). The potential for sympatry of *L. braziliensis* and *L. peruviana* appears to be restricted ecologically to the Eastern slopes of the Andes in open valleys facing the Amazonian forest, such as Huanuco (Fig. 1, HN), and

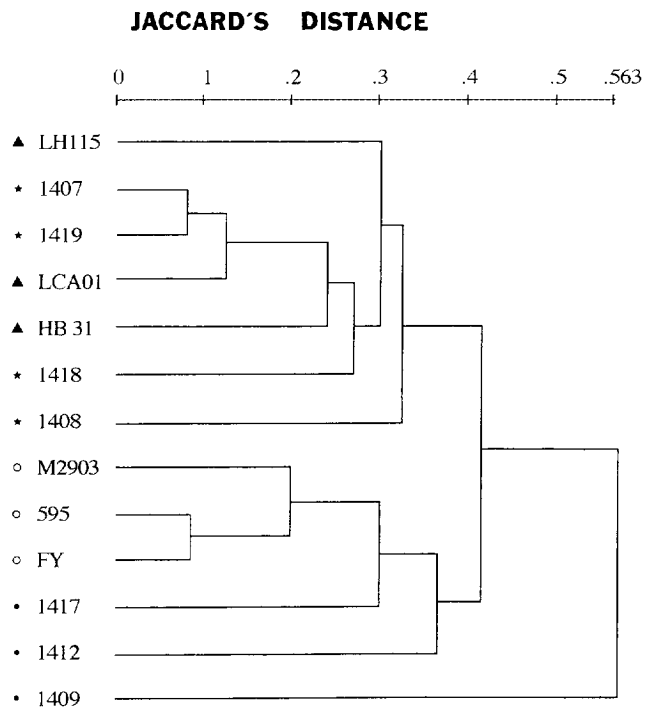

Fig. 6. UPGMA dendrogram built up from the matrix of Jaccard distances estimated from data of RAPD with primers A2, A4 and A10. (see Fig. 3 for symbols).

in the North of Peru on the Ecuadorian border where Huancabamba (Fig. 1, HB) is the only natural pass between the Amazonian forest and the Pacific Coast. This latter area was already sampled during a North-South transectional study along the Western (Pacific side) Andes, and revealed only parasites of the same karyodeme of *L. peruviana*, a population shown to be karyotypically much closer to *L. braziliensis* than to southern *L. peruviana* populations (Dujardin et al., 1993b).

During an outbreak of tegumentary leishmaniasis in the Huanuco Valley, co-existence of uta and sylvatic leishmaniasis was addressed and 7 isolates from cutaneous lesions were collected for the present study, including a case (LC1408) with mucosal involvement. Molecular characterization involved comparison of karyotyping (Dujardin et al., 1993a and b) to enzyme analysis (Ben Abderrazak et al., 1993) and evaluation of RAPD (Tibayrenc et al., 1993).

With MLEE, Huanuco isolates, when compared to reference organisms, clustered either as *L. braziliensis* (3 isolates) or as a single group (4 isolates, including LC1408) distinct from both *L. braziliensis* and *L. peruviana* (Fig. 3). This latter group displayed a typical heterozygous profile with the dimeric enzyme NH2 (Fig. 2), by expressing the alleles specific (Guerrini, 1993) respectively for *L. braziliensis* and for Southern *L. peruviana.*

Karyotype hybridization patterns with three genomic probes (Dujardin et al., 1993a) did confirm MLEE. Three Huanuco isolates were similar to *L. braziliensis*, with a specific single-banded pattern (Fig. 7), while the 4 NH2 heterozygotes had

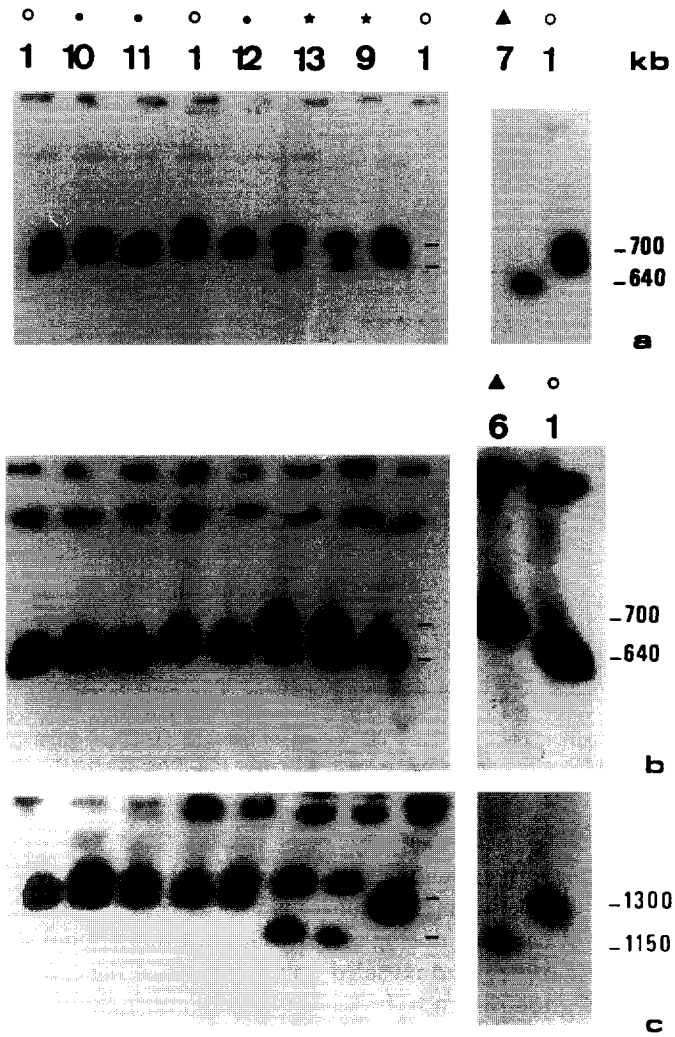

Fig. 7. Karyotype hybridization patterns of Huanuco isolates, as compared to reference stocks of *L. braziliensis* (o, lanes 1: stock M2903) and *L. peruviana* (▲, lane 7: stock LH807, lane 6: stock LCA01). Huanuco isolates characterized enzymatically as *L. braziliensis* (●) or as NH2-heterozygous *L. braziliensis*/ *L. peruviana* (★) display respectively monomorphic or heteromorphic patterns after hybridization with probes pLb-134Sp (a, pulse 65s), pLb-168 (b, pulse 65s) and pLb-22 (c, pulse 115s). Lanes 10, 11, 12: monomorphic isolates LC1409, LC1412, LC1417. Lanes 13 and 9: heteromorphic isolates LC1418 and LC1408. Band sizes are given in kb.

an heteromorphic profile for the 3 chromosomes studied, with two pLb-134Sp hybridizing bands characteristic respectively for *L. braziliensis* and for *L. peruviana*.

With RAPD, similar results were encountered. Three Huanuco isolates showed with primer A2 the 1300 bp amplification fragment specific of the *L. braziliensis* reference stocks (Fig. 5). The four NH2 heterozygotes presented also that fragment plus a 320 bp fragment amplified specifically by primer A10 in *L. peruviana* reference stocks (Fig. 4).

Altogether, the three methods applied to our Huanuco isolates suggest the co-existence of *L. braziliensis* and of putative hybrids between *L. braziliensis* and *L. peruviana* in the Huanuco Valley. NH2 heterozygous profiles as well as pLb-22 and pLb-168 heteromorphic patterns are also compatible with the hypothesis of genetic exchange between Northern and Southern *L. peruviana*. The hybrid hypothesis is supported by (i) the number (6/7) of characters of *L. braziliensis* and *L. peruviana* respectively, shared by the 4 Huanuco isolates and (ii) the fact that the shared characters are incompatible with the possibility of mixed populations; indeed we should observe 2 bands only for NH2 (dimeric enzyme), 2 bands for MPI, and an exact juxtaposition of the RAPD patterns, which was not the case. The fact that the MPI isoenzyme character of the putative hybrids is not a combination of the parental characters does not reject the hybridization hypothesis. Indeed, nature and mechanisms of hybridization in *Leishmania* are still unknown. These genetic exchanges may not involve the whole genome and might not follow a Mendelian pattern of inheritance. This could account for the result obtained from the MPI locus in the putative hybrids, which show the pattern of *L. peruviana* only. Similar results were reported by Evans et al. (1987): putative hybrids showed only 4 heterozygous enzyme profiles out of 8 polymorphic ones. Another explanation is that hybrids simply do not represent a mere F1 generation as in this case various combinations of parental characters are expected.

Hybridization patterns in *Leishmania*, first reported by Evans et al. (1987) in the Old World, have been also observed in the New World (Darce et al., 1991; Bañuls, 1993; Belli et al., 1994). Nevertheless, the frequency of genetic exchange in natural populations (Tibayrenc et al., 1990) and in experimental crosses (Panton et al., 1991) of *Leishmania* seems to be very low. The conflict between the two lines of evidence may not be real: the occasional observation of hybrids says little about the actual frequency of the phenomenon in natural conditions. The high proportion of hybrids encountered in Huanuco (4/7 isolates) can be explained by hybridization events followed by clonal propagation of the hybrids. Furthermore, propagation of some genotypes might be favoured by the epidemic character of the situation in Huanuco. These factors together with the limited size of our sampling might also explain the absence of one of the putative parents (homozygous *L. peruviana*) in Huanuco. Further studies involving more extensive samplings are hence required in order to evaluate the actual impact of hybridization phenomena on natural populations of *Leishmania*.

Comparison of the results obtained in Huanuco and other Peruvian Andean areas illustrate 2 contrasting mechanisms leading to chromosomal polymorphism. The recombination suggested in Huanuco constitutes a first and apparently minor mechanism. The second and more important mechanism was described in Western Andean valleys, and consists of chromosomal rearrangements probably selected by the environmental pressure (Dujardin et al., 1993b). This mechanism may give apparent pictures of recombination: heteromorphic chromosomal patterns, but homozygous isoenzyme and RAPD patterns (Dujardin et al., 1993b; Bañuls, 1993). A third potential mechanism might be the pseudo-sexual process proposed by Cruz et al. (1993): chromosomal polymorphism could be induced when an heteromorphic parasite would pass through an aneuploid or polyploid stage and generate all the possible combinations between the chromosomal variants during its reversion to diploidy.

The finding of distinct parasitic populations in Huanuco and their identity might be of epidemiological importance. Obviously, the presence of *L. braziliensis* in Huanuco should forewarn clinicians about the risk of espundia in some of their patients. More importantly, the heterozygous identity at this stage of a second population of parasites stresses the need for a follow-up of infected patients. Evolution of their infection might lead to classical uta if, based on their single MPI profile the infecting parasites are considered as *L. peruviana*. However, since the heterozygotic population presents genetic characters from both *L. peruviana* and *L. braziliensis*, they may have a potential for mucosal metastasis, a risk suggested in this study by the mucosal patient infected with heterozygote LC1408. Meanwhile, it should be kept in mind that the genetic makeup of the parasite is not the only factor responsible for the type of lesion: the immunogenetic background of the patient also plays an important role (Walton and Valverde, 1977), a still open question.

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